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The P2Y$_2$ nucleotide receptor requires interaction with αv integrins to access and activate G$_{12}$

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Summary

The P2Y$_2$ nucleotide receptor (P2Y$_2$R) interacts with αv integrins to activate G$_o$ and induce chemotaxis in human 1321N1 astrocytoma cells. In this study, it was determined that the P2Y$_2$R also requires interaction with αv integrins to activate G$_{12}$ and associated signaling pathways that control chemotaxis in 1321N1 cells. Mutation of the Arg-Gly-Asp (RGD) integrin-binding sequence in the first extracellular loop of the human P2Y$_2$R to Arg-Gly-Glu (RGE), which prevents integrin interaction, did not inhibit G$_q$ or ERK1/2 signaling by the P2Y$_2$R agonist UTP but completely inhibited activation of G$_{12}$ and G$_{12}$-mediated events, including Rho activation, cofilin and myosin light chain-2 phosphorylation, stress fiber formation and chemotaxis towards UTP. The involvement of G$_{12}$ in all these events was verified by using a dominant negative Gα$_{12}$ construct. G$_{12}$ activation by the P2Y$_2$R also was inhibited by anti-αvβ5 integrin antibodies and αv integrin antisense oligonucleotides, suggesting that αv integrin activity and expression are required for the P2Y$_2$R to activate G$_{12}$. Coimmunoprecipitation experiments confirmed that Gα$_{12}$ protein associates with the wild-type P2Y$_2$R and with αv integrins but not with the RGE mutant P2Y$_2$R or with α3 integrins. Collectively, these results suggest that αv integrin complexes provide the P2Y$_2$R with access to G$_{12}$, thereby allowing activation of this heterotrimeric G protein that controls actin cytoskeletal rearrangements required for chemotaxis.

Keywords

P2Y$_2$ receptor; G$_{12}$; αv integrin; Cell migration; Stress fibers

Introduction

Chemotaxis is the movement of a cell towards a chemoattractant or away from a chemorepellant and is a fundamental feature of eukaryotic cells; it is important for many physiological and pathological processes, such as embryogenesis, neurogenesis, angiogenesis, wound healing and homing of leukocytes to a site of infection. The ability of a cell to undergo chemotaxis requires the cell to assume a polarized morphology that is controlled by cell surface receptors that activate the Rho family of small GTPases, including Cdc42, Rac and Rho (Burridge and Wennerberg, 2004). Upon activation of a chemoattractant receptor, Cdc42 and Rac localize at the leading edge of a cell and control directional cell movement and the formation of a lamellipodium, respectively (Burridge and Wennerberg, 2004). Rho localizes at the rear and sides of a cell and controls the formation of contractile actin-myosin stress fibers (Xu et al., 2003). Together, these GTPases promote cell migration towards a chemoattractant by

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mediating extension of the actin cytoskeleton at the front edge of the cell and retraction of the cytoskeleton at the rear.

A variety of compounds can act as chemoattractants by stimulating Rho family GTPases: these include growth factors that activate tyrosine kinase receptors, extracellular matrix proteins that activate integrins, and compounds that activate certain G-protein-coupled receptors (GPCRs) (Burridge and Wennerberg, 2004). Recent studies have shown that GPCRs activate Rac and Rac-dependent lamellipodia formation through $G_{i/o}$, whereas the activity of Rho and Rho-dependent stress fiber formation is controlled by $G_{12/13}$ (Hart et al., 1998; Kozasa et al., 1998; Xu et al., 2003). Furthermore, studies have shown that the βγ subunits of $G_{i/o}$ are responsible for activation of Rac guanine nucleotide exchange factors (RacGEFs), which, in turn, activate Rac, whereas the α subunits of $G_{12/13}$ are responsible for activation of RhoGEFs (Neptune et al., 1999; Welch et al., 2002).

In this study, we examined the mechanism of Rho activation and Rho-dependent stress fiber formation mediated by the $P2Y_2$ nucleotide receptor ($P2Y_2R$), an integrin-associated GPCR activated by ATP or UTP that mediates stress fiber formation and chemotaxis in a variety of cell types (Bagchi et al., 2005; Kaczmarek et al., 2005; Satterwhite et al., 1999; Sauzeau et al., 2000; Wang et al., 2005) and plays an important role in monocyte (Seye et al., 2002) and neutrophil homing (Chen et al., 2006) to injured or infected tissues. Previously, we found that an RGD integrin-binding domain in the first extracellular loop of the $P2Y_2R$ enables the receptor to interact with αvβ3 and αvβ5 integrins and this interaction is prevented by mutation of the RGD sequence to RGE (Erb et al., 2001). The RGD domain also was found to be necessary for coupling of the $P2Y_2R$ to G_o-mediated but not G_q-mediated Ca^{2+} signaling (Erb et al., 2001) and a recent study by our group indicated that the $P2Y_2R$ requires interaction with αv integrins to activate G_o and to initiate G_o-mediated events, including activation of Rac and the RacGEF Vav2, upregulation of vitronectin expression and increased cell migration (Bagchi et al., 2005). Here we report that αv integrin interaction is required for the $P2Y_2R$ to access and activate G_{12}, leading to Rho activation and Rho-dependent stress fiber formation.

Results

$P2Y_2R$-mediated Rho activation and stress fiber formation require interaction with αvβ5 integrin

To determine whether integrin interaction with the $P2Y_2R$ is important for receptor signaling, we mutated the Arg95-Gly96-Asp97 (RGD) sequence in the $P2Y_2R$ to Arg95-Gly96-Glu97 (RGE), a sequence that does not have high affinity for integrins (Erb et al., 2001), and compared signaling events mediated by the wild-type and RGE mutant receptors that were expressed in human 1321N1 astrocytoma cells. These cells are devoid of endogenous G-protein-coupled P2Y receptors (Parr et al., 1994) and, thus, provide a suitable null background for this study. Also, we incorporated a hemagglutinin (HA) epitope tag at the C-termini of the wild-type and RGE mutant $P2Y_2Rs$ and used flow cytometry to verify that the cell surface expression levels of these receptor constructs were equivalent (Fig. 1A). We found that mutation of the RGD sequence to RGE did not prevent ERK1/2 phosphorylation in response to the $P2Y_2R$ agonist UTP (Fig. 1B) but completely prevented Rho activation (Fig. 1B) and stress fiber formation (Fig. 1C) induced by UTP at all concentrations tested (100 nM to 2 mM). A UTP dose-response curve indicated that the EC_{50} value for UTP-induced Rho activation by the wild-type $P2Y_2R$ was ~1 μM (supplementary material Fig. S1), which is similar to the EC_{50} value of UTP for activation of other $P2Y_2R$-mediated responses (Erb et al., 2001). These results suggest that integrin interaction with the $P2Y_2R$, via the RGD integrin-binding domain, is necessary for the $P2Y_2R$ to activate Rho and cause stress fiber formation. Likewise, Rac activation and chemotaxis mediated by the $P2Y_2R$ in 1321N1 cell transfectants were found to require expression of the RGD integrin-binding domain in the $P2Y_2R$ (Bagchi et al., 2005). As

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expected, untransfected 1321N1 cells that do not express P2Y receptors do not form stress fibers, undergo chemotaxis or exhibit Rho or Rac activation in response to the $P2Y_2R$ agonists ATP or UTP (data not shown).

We have found that the RGE mutant $P2Y_2R$ can fully stimulate ERK1/2 phosphorylation (Fig. 1B) and G_q-mediated calcium signaling (Erb et al., 2001); however, these responses require agonist concentrations that are three orders of magnitude higher than for the wild-type receptor. This has raised questions as to whether the RGE mutation affects $P2Y_2R$ signaling by preventing integrin interaction or by affecting $P2Y_2R$ agonist binding affinity. For example, a recent study by Qi et al. confirmed that mutation of the RGD sequence to RGE in the $P2Y_2R$ decreases agonist potency for inositol phosphate formation by 1000-fold but mutation of the RGD sequence to AHN did not alter agonist potency, leading the authors to speculate that the RGD to RGE mutation was affecting agonist binding affinity (Qi et al., 2005). To verify that the loss of Rho signaling observed for the $P2Y_2R$ RGE mutant was due to decreased integrin binding and not to alteration in agonist binding affinity we constructed a $P2Y_2R$ mutant in which the entire RGD integrin-binding sequence was substituted with three alanines (AAA). We found that the AAA mutant $P2Y_2R$ had a similar UTP dose response for ERK1/2 phosphorylation as the wild-type receptor (Fig. 2A) but was unable to activate Rho in response to UTP (Fig. 2B). Interestingly, 1321N1 cells expressing the AAA mutant did display a higher basal level of ERK1/2 phosphorylation and Rho activity compared with cells expressing the wild-type $P2Y_2R$ (Fig. 2A,B). The reason for the increased basal activity exhibited by the AAA mutant is unknown; nonetheless, these studies support the hypothesis that integrin interaction via the RGD domain of the $P2Y_2R$ is important for controlling Rho activation by the $P2Y_2R$.

To further assess the role of $P2Y_2R$-integrin interaction in UTP-induced Rho activation and stress fiber formation mediated by the wild-type $P2Y_2R$, we used function-blocking antibodies directed against αvβ5, an integrin that interacts with the $P2Y_2R$, or α3, an integrin that does not interact with the $P2Y_2R$ (Erb et al., 2001). Flow cytometry experiments indicate that 1321N1 cells express immunodetectable cell surface αv, α3 and β5 integrin subunits, but not β3 (Bagchi et al., 2005). Pretreatment of $P2Y_2R$-expressing cells with anti-αvβ5 integrin antibodies inhibited UTP-induced Rho activation (Fig. 3A) and stress fiber formation (Fig. 3B), whereas anti-α3 integrin antibodies had no effect (Fig. 3A,B), suggesting that αv integrin activity is important for $P2Y_2R$-mediated Rho activation and stress fiber formation. By contrast, anti-αvβ5 integrin antibodies did not inhibit Rho activation induced by fetal bovine serum (FBS) in 1321N1 cells (supplementary material Fig. S2), suggesting that αv integrins are not involved in Rho activation by growth factors present in serum.

Integrin-$P2Y_2R$ interaction regulates Rho-dependent signaling

Activation of Rho leads to stress fiber formation by causing the phosphorylation of myosin light chain-2 (MLC-2) and cofilin, an actin-depolymerizing protein that is inhibited when phosphorylated on Ser3 (Kimura et al., 1996; Moriyama et al., 1996). To verify that integrin-$P2Y_2R$ interaction is important for Rho signaling, we analyzed Rho-dependent signaling events in 1321N1 cells expressing wild-type $P2Y_2R$ or RGE mutant. UTP caused a dose-dependent increase in phosphorylation of MLC-2 (Fig. 4A) and cofilin (Fig. 4B) in cells expressing the wild-type $P2Y_2R$ but not in cells expressing the RGE mutant. Moreover, cofilin phosphorylation mediated by the wild-type $P2Y_2R$ was inhibited by pretreatment of the cells with anti-αvβ5 antibodies but not anti-α3 integrin antibodies (Fig. 4C), further demonstrating that the $P2Y_2R$ interacts selectively with αv integrins to activate Rho-mediated signaling events. Since cofilin phosphorylation can be regulated by proteins other than Rho, including Rac and testicular protein kinase 1 (Burridge and Wennerberg, 2004; Toshima et al., 2001), we used the ROCK-Rho pathway inhibitor Y-27632 to determine whether cofilin phosphorylation mediated by the $P2Y_2R$ occurs through the Rho signaling pathway. UTP-

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induced cofilin phosphorylation was completely inhibited when the cells were pretreated with Y-27632 (Fig. 4D). Furthermore, pretreatment of the cells with the $G_{i/o}$ inhibitor pertussis toxin (PTX) did not inhibit UTP-induced cofilin phosphorylation (Fig. 4D) but did inhibit UTP-induced Rac activation (Bagchi et al., 2005), suggesting that G_o-mediated activation of Rac is not involved in cofilin phosphorylation by the $P2Y_2R$.

Activation of $G\alpha_{12}$ by the $P2Y_2R$ requires interaction with αv integrin

Rho activation and Rho-dependent stress fiber formation mediated by GPCRs are controlled by heterotrimeric G proteins in the $G_{12/13}$ family (Buhl et al., 1995; Xu et al., 2003). Generally, GPCRs that stimulate stress fiber formation also couple to $G_{q/11}$ but regulate stress fiber assembly through activation of either G_{12} or G_{13} (Gohla et al., 1999). Here, we directly investigated whether the RGD integrin-binding domain of the $P2Y_2R$ is required for activation of specific G proteins (i.e. G_{12} and G_q). Results indicated a 2.5-fold increase in $[^{35}S]GTP\gamma S$ binding to $G\alpha_{12}$ immunoprecipitated from UTP-treated membrane extracts of 1321N1 cells expressing the wild-type $P2Y_2R$ compared with untreated controls, but extracts from cells expressing the RGE mutant receptor did not exhibit an increase in $[^{35}S]GTP\gamma S$ binding to $G\alpha_{12}$ in response to UTP (Fig. 5A). By contrast, UTP induced a two- to threefold increase in $[^{35}S]GTP\gamma S$ binding to $G\alpha_q$ upon activation of either the wild-type or RGE mutant $P2Y_2R$ (Fig. 5B). Activation of $G_{12/13}$ and $G_{q/11}$ proteins by the $P2Y_2R$ was also verified by analyzing serine or threonine phosphorylation of Gα12/13 (Kozasa and Gilman, 1996) and tyrosine phosphorylation of Gαq/11 (Umemori et al., 1997), as previously described. We found that UTP caused phosphorylation of both $G\alpha_{12}$ and $G\alpha_q$ in 1321N1 cells expressing the wild-type $P2Y_2R$ (Fig. 5C), whereas no phosphorylation of $G\alpha_{13}$ was detected in these cells (data not shown). UTP caused phosphorylation of $G\alpha_q$ but not $G\alpha_{12}$ in cells expressing the RGE mutant $P2Y_2R$ (Fig. 5C), suggesting that αv integrin interaction with the $P2Y_2R$ is required for UTP-induced activation of G_{12} but not $G_{q.}$

To further assess whether αv integrins are involved in $P2Y_2R$-mediated activation of $G\alpha_{12}$, we tested the effects of inhibition of αv activity or expression using anti-αv integrin antibodies or αv antisense oligonucleotides, respectively. We found that $G\alpha_{12}$ phosphorylation by UTP was inhibited by pretreatment with anti-αv but not with anti-α3 integrin antibodies in 1321N1 cells expressing the wild-type $P2Y_2R$ (Fig. 6A). Likewise, transfection of αv antisense oligonucleotides in 1321N1 cells expressing the wild-type $P2Y_2R$, which significantly suppressed αv expression (Fig. 6B), completely inhibited $G\alpha_{12}$ activation by UTP, as assessed by GTPγS binding (Fig. 6B). Transfection of these cells with αv sense oligonucleotides did not inhibit $G\alpha_{12}$ activation by UTP (Fig. 6B). Together, these results suggest that αv integrin expression and activity are required for $P2Y_2R$-$G\alpha_{12}$ coupling.

$P2Y_2R$ accesses $G\alpha_{12}$ in a complex with αv integrins

To determine whether the $P2Y_2R$ interacts with $G\alpha_{12}$ in a complex with αv integrins, co-immunoprecipitation experiments were performed. Results indicated that endogenous αv integrin co-immunoprecipitated with the HA-tagged wild-type $P2Y_2R$ to a much greater extent than with the HA-tagged RGE mutant $P2Y_2R$ expressed in 1321N1 cells (Fig. 7A). This association between αv integrin and the wild-type $P2Y_2R$ occurred with or without activation of the $P2Y_2R$, although 5 min stimulation with UTP did cause a slight but reproducible reduction in association between these proteins (Fig. 7A). We also found that endogenous $G\alpha_{12}$ coimmunoprecipitated with the wild-type $P2Y_2R$ but not with the RGE mutant, whereas endogenous $G\alpha_q$ coimmunoprecipitated with both $P2Y_2R$ constructs (Fig. 7A). Co-immunoprecipitation of both $G\alpha_q$ and $G\alpha_{12}$ with the wild-type $P2Y_2R$ was inhibited after UTP treatment (Fig. 7A), consistent with the concept that activation of GPCRs causes the release of receptor-coupled G protein subunits, thus triggering various downstream responses. Although we were unable to detect any association between $G\alpha_{12}$ and αv integrins when

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endogenous levels of these proteins were used, we did find that wild-type $G\alpha_{12}$ overexpressed in 1321N1 cells coimmunoprecipitated with endogenous α_v integrins but not with endogenous α3 integrins (Fig. 7B), suggesting that G_{12} selectively associates with complexes containing αv integrins. Interestingly, UTP treatment did not cause dissociation of $G\alpha_{12}$ and αv integrins (Fig. 7B) and fluorescence microscopy images indicated that UTP treatment caused a slight redistribution of $G\alpha_{12}$ and $G\alpha_o$ onto membrane protrusions or lamellipodia in 1321N1 cells (Fig. 8).

$G\alpha_{12}$ activity is required for $P2Y_2R$-mediated stress fiber formation and cell migration

To verify that the G_{12} protein is responsible for $P2Y_2R$-mediated Rho activation and downstream signaling events, a dominant negative mutant of $G\alpha_{12}$ ($G\alpha_{12}$DN, Q231L/D299N) was used (Yang et al., 2005). Overexpression of $G\alpha_{12}$DN in 1321N1 cells expressing the wild-type $P2Y_2R$ completely inhibited UTP-induced Rho activation, cofilin phosphorylation, stress fiber formation and cell migration (Fig. 9A,C,D), but did not inhibit UTP-induced ERK1/2 phosphorylation (Fig. 9B), indicating that G_{12} is specifically required for $P2Y_2R$-mediated Rho activation and Rho-dependent signaling events leading to stress fiber formation and ultimately, cell migration.

Discussion

The G-protein-coupled $P2Y_2R$ is known to activate several heterotrimeric G proteins, including G_o and G_q (Boarder et al., 1995; Erb et al., 2001). In this study, we show for the first time that the $P2Y_2R$ is also able to activate G_{12} and to initiate chemotactic signaling events downstream of G_{12}, including Rho activation, cofilin phosphorylation, stress fiber formation and directional cell migration (Figs 1, 5 and 9). Furthermore, we demonstrate here and in previous studies that an RGD integrin-binding domain in the first extracellular loop of the $P2Y_2R$ is necessary for the $P2Y_2R$ to activate G_o and G_{12}, but not G_q (Fig. 5) (Bagchi et al., 2005; Erb et al., 2001), suggesting that integrin complexes provide the $P2Y_2R$ with access to select pools of heterotrimeric G proteins. Since the $P2Y_2R$ is known to interact with αv integrins (Erb et al., 2001), we performed a series of coimmunoprecipitation experiments to verify that this interaction is required for the $P2Y_2R$ to access G_{12} protein. These experiments showed that (1) $G\alpha_{12}$ coimmunoprecipitated with αv integrins but not with α3 integrins; (2) the $P2Y_2R$ coimmunoprecipitated with $G\alpha_q$, $G\alpha_{12}$ and αv integrins; and (3) mutation of the $P2Y_2R$ integrin-binding domain (i.e., substitution of RGD with RGE that does not bind integrins) did not affect the ability of $P2Y_2R$ to co-immunoprecipitate with $G\alpha_q$ but inhibited $P2Y_2R$ co-immunoprecipitation with $G\alpha_{12}$ and αv integrins (Fig. 7). Although many studies suggest that amino acids located in intracellular loop 2 and the N- and C-terminal portions of intracellular loop 3 are the key elements responsible for GPCR selectivity of G protein recognition (Wess, 1997), the results presented here suggest that αv integrin complexes are also important for establishing interaction between select G proteins and a GPCR. And, although it is well known that GPCRs require integrin activity to stimulate chemotaxis (Miettinen et al., 1998; Till et al., 2002), this is the first indication that a GPCR requires interaction with an integrin to provide access to specific heterotrimeric G proteins that regulate chemotaxis.

Similar to GPCR-mediated chemotaxis, there is some evidence that growth factor receptors and integrins use heterotrimeric G proteins to stimulate chemotaxis. Pertussis toxin (PTX), which specifically inactivates $G_{i/o}$ proteins by covalent modification of the α subunits, has been found to inhibit VEGF-induced monocyte migration (Barleon et al., 1996) as well as growth-factor-induced Rac1 and Cdc42 activation by a chimeric EGF/VEGF receptor (Zeng et al., 2002). In addition, the latter study determined that overexpression of the Gβγ-sequestering minigene, hβARK1, inhibited growth-factor-induced Rac1 and Cdc42 activation, suggesting that βγ subunits of PTX-sensitive $G_{i/o}$ proteins are involved in growth-factor-induced

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activation of Rac and Cdc42 (Zeng et al., 2002). Vitronectin-induced chemotaxis of human melanoma cells mediated by the αvβ3 integrin is also inhibited by PTX (Aznavoorian et al., 1996), suggesting a role for $G_{i/o}$ proteins in this process. Although the mechanism of heterotrimeric G protein activation by growth factor receptors and integrins is unclear, it is possible that these chemoattractant receptors stimulate the release of ATP, thus triggering activation of G-protein-coupled nucleotide and nucleoside receptors involved in chemotaxis. In support of this idea, studies have demonstrated that ATP is released from epithelial cells (McNamara et al., 2006; McNamara et al., 2001) and migrating neutrophils (Chen et al., 2006) upon exposure to chemotactic bacterial proteins.

Recently, the general importance of the $P2Y_2R$ and the adenosine A3 receptor in mediating chemotaxis towards bacterial proteins was demonstrated in neutrophils (Chen et al., 2006). The authors showed that ATP is released at the leading edge of human neutrophils migrating towards the bacterial chemoattractant N-formyl-Met-Leu-Phe (FMLP) and is rapidly broken down to adenosine by ecto-ATPases on the cell surface. In vivo assessment of neutrophil infiltration into the peritoneal cavity of $P2Y_2R^{-/-}$ and A3 receptor$^{-/-}$ mice injected with a murine chemotactic protein (Trp-Lys-Tyr-Met-Val-Met-NH_2) or with *Staphylococcus aureus* bacteria indicated that both the $P2Y_2R$ and the adenosine A3 receptor are required for neutrophil recruitment. Furthermore, neutrophils lacking the adenosine A3 receptor migrated toward Trp-Lys-Tyr-Met-Val-Met-NH_2, but with diminished speed, whereas neutrophils lacking the $P2Y_2R$ showed a loss in sensing of the chemoattractant gradient. In agreement with the work of Chen et al. on neutrophils, we found that the $P2Y_2R$ remains evenly distributed in the plasma membrane of 1321N1 cells after receptor activation (supplementary material Movie 1), which supports the conclusion that the $P2Y_2R$ controls chemotaxis by sensing and amplifying signals induced by chemoattractants.

Results presented in this study demonstrate that $G\alpha_{12}$ selectively associates with complexes containing αv integrins (Fig. 7). Although the mechanism of this interaction is unclear, it is possible that the cadherin family of cell-surface adhesion proteins may be involved because $G\alpha_{12}$ has been found to interact with the cytoplasmic tails of several cadherins (Meigs et al., 2001) and E-cadherin is known to associate with αv integrins (von Schlippe et al., 2000). Another mechanism of αv-$G\alpha_{12}$ association may involve Tec tyrosine kinases. Members of the Tec family have been found to interact directly with $G\alpha_{12}$ and with focal adhesion kinase (Chen et al., 2001;Jiang et al., 1998), thus linking G_{12} to integrin complexes. Furthermore, $G\alpha_{12}$ can interact directly with leukemia-associated RhoGEF (LARG) and, upon phosphorylation of LARG by Tec, $G\alpha_{12}$ effectively stimulates the RhoGEF activity of LARG (Suzuki et al., 2003). Although we were unable to detect LARG expression in 1321N1 cells (data not shown), LARG belongs to a subfamily of RhoGEFs (including Lsc/p115 RhoGEF and PDZ-RhoGEF), which, unlike other RhoGEFs, contains a regulator of G protein signaling (RGS) domain that facilitates binding to $G\alpha_{12/13}$ (Francis et al., 2006;Fukuhara et al., 2000;Fukuhara et al., 1999;Reuther et al., 2001) and, in some instances, $G\alpha_q$ (Booden et al., 2002;Vogt et al., 2003). Members of this RhoGEF subfamily share the ability to specifically activate RhoA but not other Rho family GTPases, such as Rac1 and Cdc42 (Banerjee and Wedegaertner, 2004). Therefore, the link between this subfamily of RhoGEFs and the $P2Y_2R$ warrants further investigation to better define how $P2Y_2Rs$ access G_{12} in αv-containing complexes.

In summary, the present study indicates that the $P2Y_2R$ requires interaction with αv integrins to access G_{12}, but not G_q, and to stimulate chemotactic signaling events mediated by G_{12}, including Rho activation, cofilin and MLC-2 phosphorylation and stress fiber formation. Since our previous work indicated that the $P2Y_2R$ also requires interaction with αv integrins to activate G_o and G_o-mediated cell migration (Bagchi et al., 2005), these studies establish that

αv integrin complexes are required for the $P2Y_2R$ to access select heterotrimeric G proteins involved in chemotaxis.

Materials and Methods

Materials

Anti-human αv (Q20), αvβ5 (P1F76) and α3 (Ralph 3.2) monoclonal Abs, mouse IgG, polyclonal rabbit anti-human $G\alpha_{12}$, anti-human $G\alpha_{q/11}$, and anti-human ERK1/2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit anti-human cofilin, anti-phospho-cofilin, anti-phospho-MLC-2, and anti-phospho-ERK1/2 antibodies were purchased from Cell Signaling (Beverly, MA). The polyclonal rabbit anti-human actin antibodies were purchased from Cytoskeleton (Denver, CO). The mouse antiphosphoserine/threonine antibody and the anti-α_v integrin antibody for immunoblot analysis were purchased from BD Bioscience (San Jose, CA). The mouse anti-phosphotyrosine antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-HA conjugated agarose beads and anti-HA antibody were purchased from Covance (Berkeley, CA). Oregon-Green-conjugated phalloidin, Rhodamine-conjugated phalloidin and Texas-Red-conjugated DNase I were purchased from Molecular Probes (Eugene, OR). The Rho-dependent kinase inhibitor Y27632 was purchased from Calbiochem (Indianapolis, IN). All other reagents including nucleotides were obtained from Sigma-Aldrich (St Louis, MO), unless otherwise specified.

Cell culture and transfection

Human 1321N1 astrocytoma cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 5% (v/v) fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin and maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were stably transfected with cDNA encoding either the wild-type or RGE mutant $P2Y_2R$, as previously described (Erb et al., 2001). Both receptor constructs contained sequence encoding a hemagglutinin (HA) tag at the N-terminus of the $P2Y_2R$, as previously described (Erb et al., 2001). To make the AAA-$P2Y_2R$ mutant, the RGD sequence was also substituted with Ala-Ala-Ala using the QuikChange XL site-directed mutagenesis kit (Stratagene, CA), and the HA-tagged $P2Y_2R$ cDNAs were excised from pLXSN vectors by digesting with *Eco*RI and *Bam*HI, and ligated into the pcDNA3.1(-) mammalian expression plasmid. The $G\alpha_{12}$ wild type and dominant-negative construct (Q231L/D299N) were obtained from Guthrie cDNA Resource Center (Sayre, PA). Human 1321N1 cells expressing the wild-type $P2Y_2R$ were cultured to 80% confluence and transiently transfected with the $G\alpha_{12}$ constructs in the pcDNA3.1+ vector using the Lipofectamine 2000 reagent (Invitrogen, CA). The transfection efficiency for the $G\alpha_{12}$ constructs was determined to be ~60% using an indirect immunofluorescence assay. The day before experimental use of the cells, the growth medium was replaced with serum-free medium. The αv integrin was suppressed with αv antisense oligonucleotides, as described previously (Bagchi et al., 2005).

Actin stress fiber formation

Cells were plated on glass coverslips and treated as indicated at 37°C in serum-free DMEM. Then, cells were washed in PBS, fixed for 10 minutes in 3.7% (v/v) formaldehyde, treated with 0.5% (v/v) Triton X-100, and rinsed in PBS. For staining of F-actin, cells were incubated with 5 μg/ml 488 Oregon-Green-conjugated phalloidin for 45 minutes at room temperature and then washed with PBS. Texas-Red-labeled DNase I (5 μg/ml) was used to localize monomeric G-actin. In Fig. 9C, cells were incubated with rabbit anti-$G\alpha_{12}$ antibody, washed and then stained with Rhodamineconjugated phalloidin and Oregon-Greenlabeled goat anti-rabbit IgG for 45 minutes. Coverslips were mounted on glass slides in ProLong antifade reagent (Molecular Probes) and examined using fluorescence microscopy (Nikon, Eclipse TE300) at room

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temperature. The objective was a Nikon Plan Fluor 40× lens. Images were acquired and processed with Northern Eclipse 6.0 software via a QImaging camera (Qimaging, British Columbia, Canada). Single cells were selected by cropping the image.

Rho activity assay

A Rho activation assay kit (Upstate Biotechnology) was used to assess Rho activity according to the manufacturer's instructions. Briefly, cells were cultured in 100-mm tissue culture dishes in culture medium and starved with serum-free medium for 24 hours before being stimulated with UTP for 5 minutes at 37°C. Cells then were washed three times with ice-cold PBS, suspended in Lysis Buffer containing 125 mM HEPES pH 7.5, 750 mM NaCl, 5% (v/v) Igepal CA-630, 50 mM $MgCl_2$, 5 mM EDTA and 10% (v/v) glycerol, and the lysates were transferred to 1.5 ml tubes. Forty microliters of Rhotekin Rho binding domain (RBD)-agarose that only recognizes GTP-bound Rho were added to 500 μl lysate for 45 minutes at 4°C. The beads were pelleted by centrifugation (30 seconds at 14,000 ***g*** and 4°C) and washed three times with Lysis Buffer. Finally, the beads were resuspended in 40 ml of 2× Laemmli sample buffer [120 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (w/v) sucrose, 1 mM EDTA, 50 mM dithiothreitol, and 0.003% (w/v) Bromophenol Blue] and western blot analysis (see below) was performed with 1:1000 anti-Rho antibody (Upstate Biotechnology).

[^{35}S]GTPγS binding assay

Membranes (80 μg protein) from 1321N1 astrocytoma cell transfectants expressing the wild type or RGE mutant $P2Y_2R$ or the pLXSN vector were isolated, as previously described (Tian et al., 1994) and incubated in assay buffer [50 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1 μM guanosine 5′-diphosphate, 1× protease inhibitor cocktail (Roche), and 50 nCi of [^{35}S]GTPγS; 1250 Ci/mmol; Perkin Elmer, CA] containing the indicated concentration of UTP. Samples were incubated for 20 minutes at 30°C followed by addition of 0.5 ml ice-cold buffer containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 5 mM $MgCl_2$. The samples were centrifuged at 100,000 ***g*** for 15 minutes at 4°C and the resulting pellets were resuspended in 500 μl of solubilization buffer [100 mM Tris-HCl pH 7.4, 200 mM NaCl, 1 mM EDTA, 1.25% (v/v) NP40, 0.2% (w/v) SDS and 1× protease inhibitor cocktail]. Extracts were incubated overnight with 1:1000 anti-$G\alpha_{12}$ or anti-$G\alpha_q$ antibody at 4° C. The extract was then incubated with 50 μl of a 50% protein-G-agarose suspension, and the immune complexes were collected by centrifugation and washed three times in wash buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, and 5 mM MgCl). [^{35}2 S]GTPγS binding in the immunoprecipitates was quantified by liquid scintillation counting.

Immunoblot analysis and immunoprecipitation

Immunoblotting (IB) was performed as previously described (Liu et al., 2004). After the IB procedure, the membranes were stripped and reprobed with anti-actin or anti-ERK1/2 antibody to assess protein loading. Lysates from 1321N1 cell transfectants expressing the HA-tagged wild-type or RGE mutant $P2Y_2R$, or the pLXSN vector were used for immunoprecipitation (IP) with anti-HA-conjugated agarose beads, as previously described (Liu et al., 2004). IP was also performed with cell lysates and anti-$\alpha_v\beta_5$ or anti-α_3 antibody or normal goat IgG (negative control). The immune complexes were precipitated by protein-G-conjugated beads and analyzed by IB with anti-G_{12} antibody. Phosphorylation of G proteins was detected by IP of G_{12} or tyrosine-phosphorylated proteins using anti-G_{12} or anti-phosphotyrosine antibody, respectively. The immunoprecipitated samples were analyzed by IB with anti-phosphoserine/threonine or anti-G_q antibody, respectively.

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Cell migration assay

Cell migration assays were performed with 8-μm pore size Transwells (Costar) as described (Bagchi et al., 2005). In brief, the cells were cultured at 37°C for 24 hours in DMEM supplemented with 5% fetal bovine serum, suspended by trypsinization, washed and resuspended in 100 μl of serum-free DMEM (5×10^4 cells) and placed in the upper chamber of the Transwells. The lower chamber was filled with 600 μl serum-free medium with or without 100 μM UTP. The cells were allowed to migrate for 16 hours at 37°C. Cells migrating to the lower side of the membrane were fixed with cold methanol and stained with Accustain. Cells were counted in 10 microscopic fields at 20× magnification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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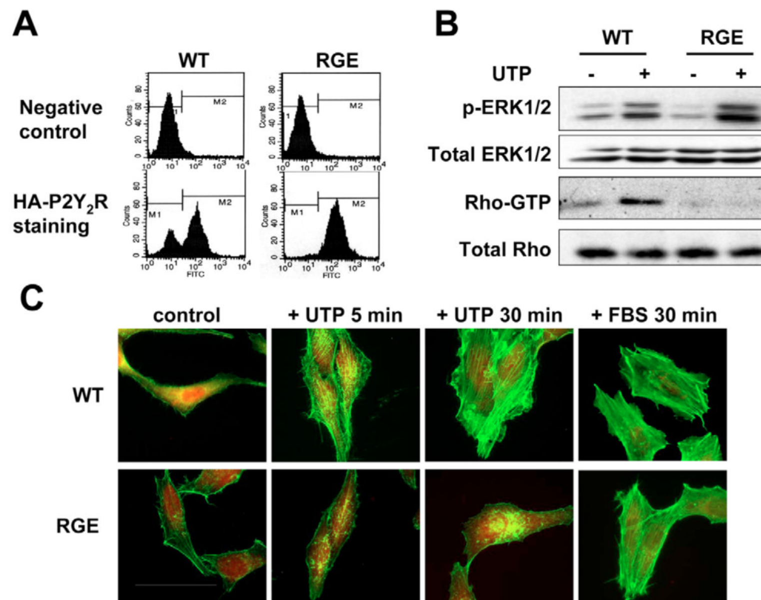

Fig. 1.

$P2Y_2R$-mediated Rho activation and stress fiber formation require $P2Y_2R$ interaction with αv integrins. (A) Cell surface expression of HA-tagged wild-type (WT) or RGE mutant (RGE) $P2Y_2Rs$ in 1321N1 cells was determined by flow cytometry. Cells stained with only secondary antibody were used as a negative control. (B) Cells expressing the WT or RGE mutant $P2Y_2R$ were treated with or without 1 mM UTP for 5 minutes prior to measuring ERK1/2 phosphorylation and Rho activity. Results shown are representative of three experiments. (C) Cells expressing the WT or RGE mutant $P2Y_2R$ were treated with or without 1 mM UTP or 20% FBS (positive control) for the indicated time and stress fibers were visualized by staining filamentous actin (shown in green) with Oregon-Green-labeled phalloidin. Texas-Red-labeled DNase I was used to label monomeric actin (shown in red). More than 100 cells were examined in three separate experiments, and representative images are shown. Bar, 50 μm.

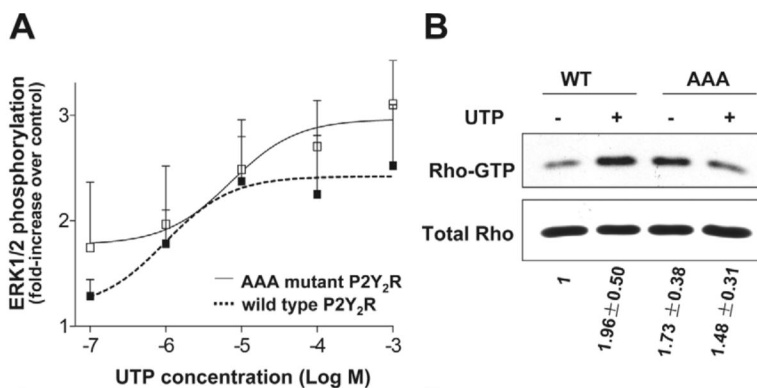

Fig. 2.

ERK1/2 and Rho activation mediated by WT and AAA mutant $P2Y_2$Rs. Human 1321N1 cells were transiently transfected with either WT (RGD) or AAA mutant $P2Y_2$Rs in pcDNA3.1(-). Transfected cells were starved overnight and stimulated with the indicated concentration of UTP for 5 minutes prior to measuring ERK1/2 phosphorylation (A) or Rho activity (B). (A) UTP-induced with 1 mM UTP for 5 minutes before measuring ERK1/2 phosphorylation is expressed as fold increase over basal level in cells transfected with WT $P2Y_2$R. Data points represent the mean ± s.e.m. of results from three experiments. (B) The Rho-GTP band density was normalized to total Rho and relative intensities are shown below each band as mean ± s.e.m. of results from four experiments.

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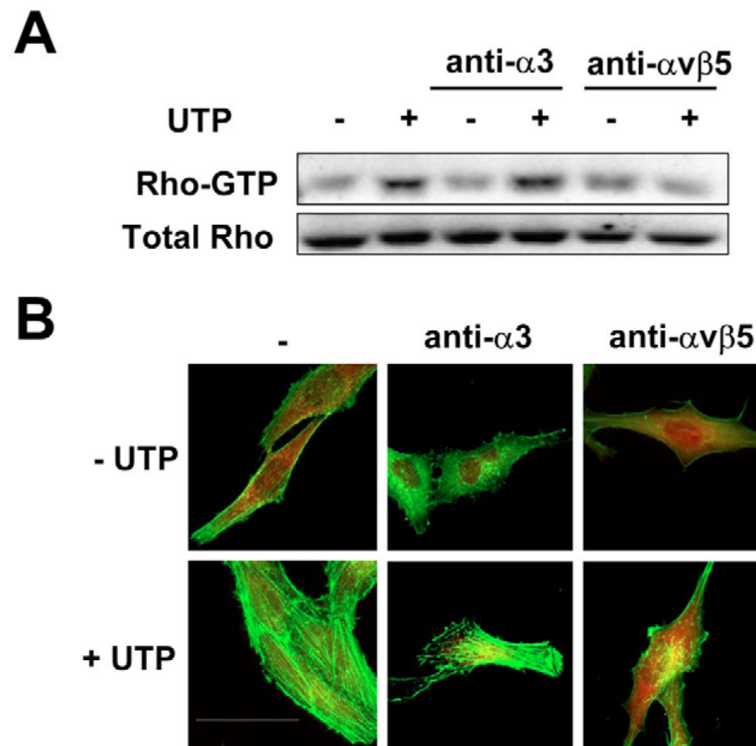

Fig. 3.

$P2Y_2R$-mediated Rho activation and stress fiber formation require αv integrin activity. Human 1321N1 cells expressing the WT $P2Y_2R$ were incubated overnight at 37°C in serum-free medium with 10 μg/ml anti-αvβ5 or anti-α3 antibody. Cells were then treated with or without 100 μM UTP for 5 minutes before analysis of Rho activity (A) or 30 minutes before analysis of stress fiber formation (B). (A) Representative blots from three experiments are shown. (B) More than 100 cells were examined in three separate experiments and representative images are shown. Bar, 50 μm.

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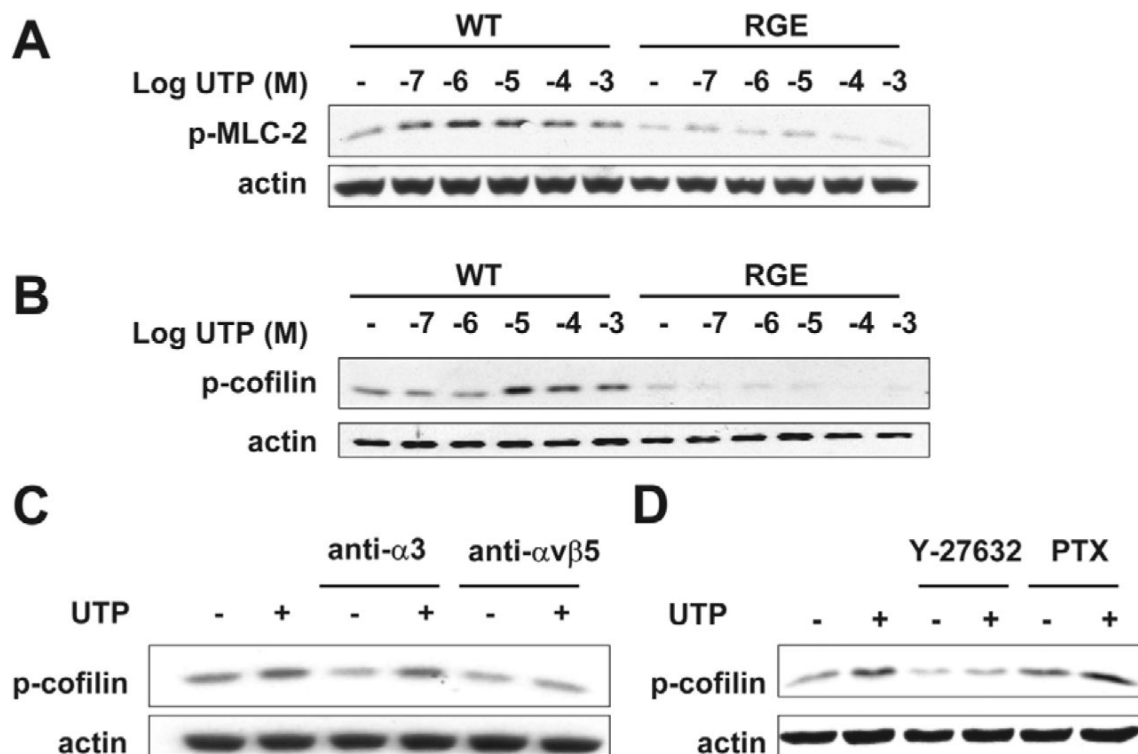

Fig. 4.

$P2Y_2R$-αv integrin interaction regulates Rho-mediated signaling. (A,B) Human 1321N1 cells expressing the WT or RGE mutant $P2Y_2R$ were incubated with the indicated concentration of UTP for 5 minutes. Cell lysates were prepared and analyzed by immunoblotting with (A) anti-phospho-myosin light chain 2 (p-MLC-2) or (B) anti-phospho-cofilin antibodies. (C) Cells expressing the WT $P2Y_2R$ were incubated overnight with or without 10 μg/ml anti-αvβ5 or anti-α3 antibody, then stimulated with 100 μM UTP for 5 minutes. Cell lysates were prepared and analyzed by immunoblotting with anti-phospho-cofilin antibodies. (D) Cells expressing the WT $P2Y_2R$ were pretreated at 37°C in serum-free medium with the Rho-dependent kinase inhibitor Y-27632 (10 μM) for 1 hour or 200 ng/ml *Bordetella pertussis* toxin (PTX) overnight, then stimulated with 100 μM UTP for 5 minutes. Cell lysates were analyzed by immunoblotting with anti-phospho-cofilin antibodies. Protein loading in each lane was evaluated by stripping the membrane of antibodies and re-probing with anti-actin antibodies. Blots representative of 3-5 experiments are shown.

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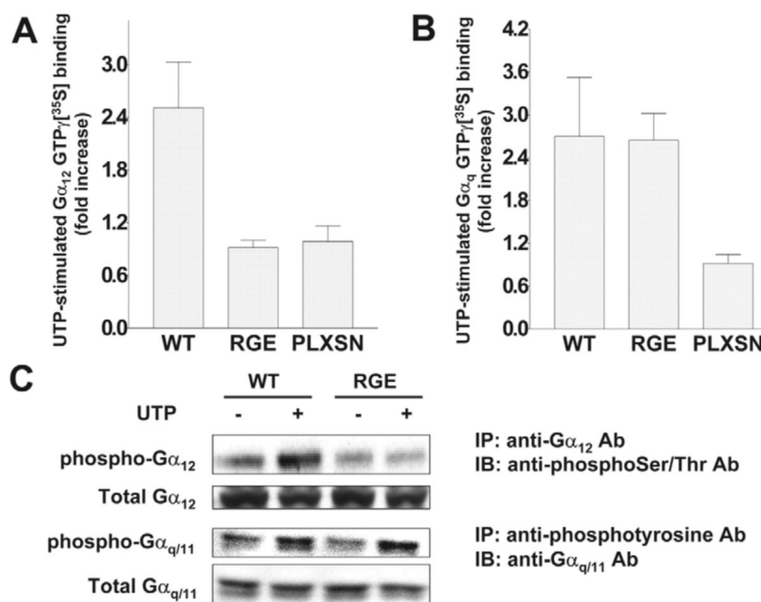

Fig. 5.

$P2Y_2R$-αv integrin interaction is required for G_{12} coupling. (A,B) Membrane preparations from 1321N1 cells expressing the WT or RGE mutant $P2Y_2R$ or pLXSN vector-transfected cells (negative control) were used in $[^{35}S]$GTPγS binding assays in the presence or absence of 1 mM UTP. After termination of the assay, samples were immunoprecipitated with antiserum against (A) $G\alpha_{12}$ or (B) $G\alpha_{q/11}$ and radioactivity in the immunoprecipitates was calculated. Data are the means ± s.e.m. of results from three separate experiments and are shown as fold increase over untreated cells. (C) Human 1321N1 cells expressing the WT or RGE mutant $P2Y_2R$ were incubated with 1 mM UTP for 2 minutes. G_{12} activation was detected by immunoprecipitation (IP) of $G\alpha_{12}$ with anti-$G\alpha_{12}$ antibody and immunoblotting (IB) of $G\alpha_{12}$ with anti-phosphoserine/threonine antibody. $G_{q/11}$ activation was detected by IP with anti-phosphotyrosine antibody and IB with anti-$G\alpha_{q/11}$ antibody. Blots representative of three experiments are shown.

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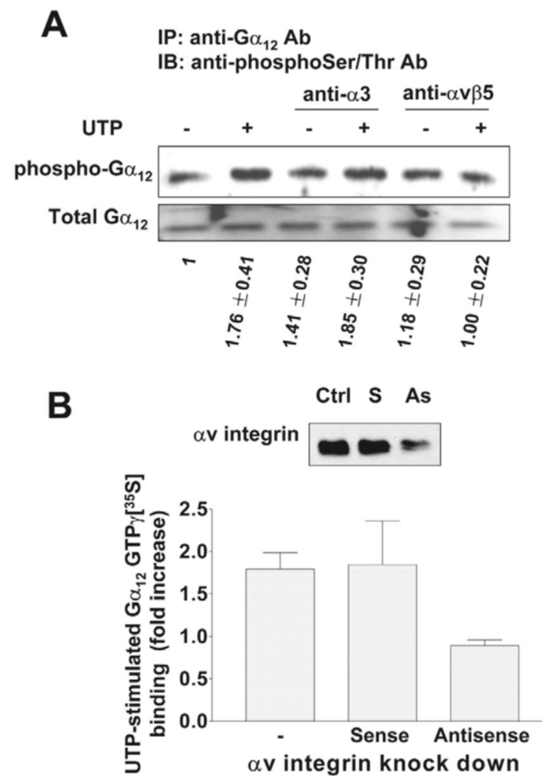

Fig. 6.

$P2Y_2R$-mediated G_{12} activation requires αv integrin expression and activity. (A) Cells expressing the WT $P2Y_2R$ were incubated overnight at 37°C in serum-free medium with 10 μg/ml anti-αvβ5 or anti-α3 antibody, then stimulated with 100 μM UTP for 2 minutes. $G\alpha_{12}$ phosphorylation was detected as described in Fig. 5. Relative intensities are shown below each band as mean ± s.e.m. of results from four experiments. (B) Cells expressing the WT $P2Y_2R$ were transfected with 5 μg of αv antisense (AS) or sense (S) oligonucleotides, as described previously (Bagchi et al., 2005). Lipofectamine transfected cells served as a negative control. Expression of αv integrin in these cells was determined by immunoblot analysis and a representative blot is shown. UTP-induced binding of $[^{35}S]GTP\gamma S$ to $G\alpha_{12}$ was determined, as described in Fig. 5. Data are the mean ± s.e.m. of results from three separate experiments and are shown as fold increase over untreated cells.

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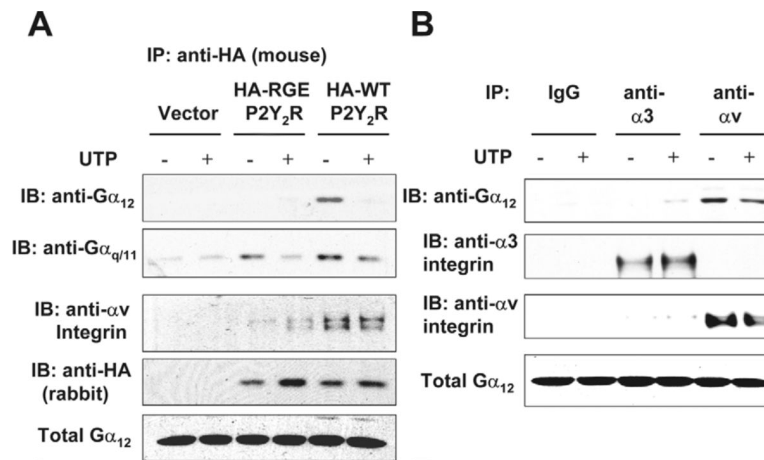

Fig. 7.

The P2Y$_2$R accesses G$_{12}$ in a complex with αv integrins. (A) RGD-dependent association of the P2Y$_2$R with αv integrins and Gα$_{12}$. Cells expressing the HA-tagged WT or RGE mutant P2Y$_2$R and endogenous G$_{q/11}$, G$_{12}$ and αv integrins were treated with or without 1 mM UTP for 5 minutes and lysates were subjected to IP with mouse anti-HA antibody conjugated to agarose beads and IB with anti-Gα$_{12}$, anti-Gα$_{q/11}$, anti-αv integrin, or anti-HA antibody. Cells transfected with the pLXSN vector served as a negative control. (B) Gα$_{12}$ associates with αv integrins. Human 1321N1 cells expressing cDNA for the WT P2Y$_2$R and Gα$_{12}$ were treated with or without 100 μM UTP for 5 minutes and lysates were subjected to IP with IgG, anti-α3 integrin, or anti-αv integrin antibody followed by IB with anti-Gα$_{12}$, anti-α3 integrin or anti-αv integrin antibody. Blots representative of three experiments are shown.

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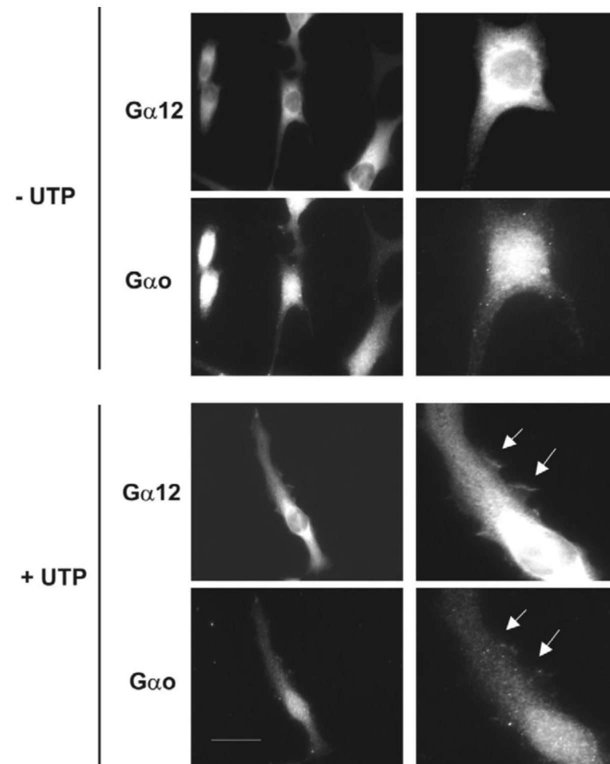

Fig. 8.

Co-immunostaining of $G\alpha_{12}$ and $G\alpha_o$ in cells exposed to a UTP gradient. Human 1321N1 cells expressing wild-type $P2Y_2Rs$ were plated on chambered coverslips and incubated overnight in 300 µl serum-free medium. UTP (1 µl of a 100 mM solution) was added at the bottom left and after 30 minutes, cells were washed, fixed, permeabilized and stained with rabbit anti-$G\alpha_{12}$ and mouse anti-$G\alpha_o$ antibodies. Texas-Red-conjugated anti-rabbit IgG and Alexa Fluor 488-conjugated anti-mouse IgG were then used to localize $G\alpha_{12}$ and $G\alpha_o$, respectively. Images representative of three experiments are shown. Arrows indicate membrane protrusions indicative of lamellipodia formation. Bars, 50 µm (left column); 20 µm (enlarged images in right column).

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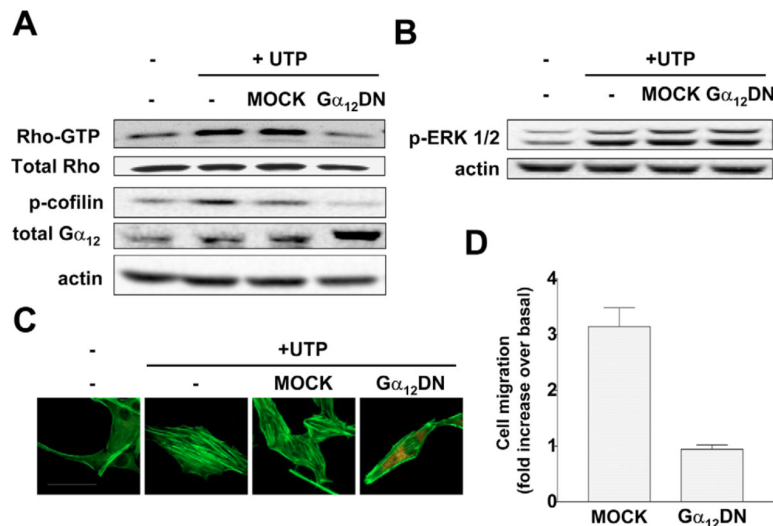

Fig. 9.

Effect of dominant-negative $G\alpha_{12}$ ($G\alpha_{12}$ DN) on signaling events mediated by the $P2Y_2R$. (A-D) Human 1321N1 cells expressing the WT $P2Y_2R$ were cultured to 80% confluence and transiently transfected with the $G\alpha_{12}$DN mutant in the pcDNA3.1+ vector. MOCK, vector transfected negative control. Transfected cells were incubated with 100 μM UTP for 5 minutes (A,B) or 30 minutes (C) and assayed for (A) Rho activity and cofilin phosphorylation, (B) ERK1/2 phosphorylation and (C) stress fiber formation, as described in the Materials and Methods. Total $G\alpha_{12}$ was detected with anti-$G\alpha_{12}$ antibody, as shown in A. Antibody-labeled $G\alpha_{12}$ in (C) is shown in red and phalloidin-labeled filamentous actin is shown in green. (D) Cells (5×10^4) transfected with the indicated constructs were seeded into the upper chamber of Transwells. Lower chambers contained serum-free medium with or without 100 μM UTP. Cell migration was evaluated 16 hours after UTP stimulation and is expressed as the fold increase in the number of cells that moved across the Transwell membranes in response to UTP as compared to untreated controls. (A,B) Blots representative of three-five experiments are shown. (C) Images representative of three separate experiments are shown. Bar, 40 μm. (D) Data shown are the mean ± s.e.m. of results from five experiments.